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Immunohistochemical distributions of cathepsin B and basement membrane antigens in human lung adenocarcinoma: association with invasion and metastasis

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Abstract The distributions of cathepsin B (CB) a lysosomal cysteine proteinase, type IV collagen (CIV) and laminin (LM), which are main components of basement membranes (BMs) were studied in a series of 64 human lung adenocarcinomas using an immunohistochemical technique. Over-expression of CB (> 80% positive cells) was significantly associated with the grade of tumour differentiation ($p < 0.01$), with lymph node metastasis ($p < 0.01$) and with BM degradation ($p < 0.01$) detected by the staining pattern of CIV and LM. It was significantly associated with a prognostic disadvantage ($p < 0.01$). The immunohistochemical staining pattern of CB has a close relationship with degradation of BM, and may be used as a marker for tumour metastasis and prognosis in lung adenocarcinoma.

Key words Cathepsin B · Basement membrane
Type IV collagen · Metastasis
Lung adenocarcinoma

Introduction

The prevalence of lung cancer is increasing year by year in many countries. However, the management of lung cancer patients, especially those with lung adenocarcinomas, is far from satisfactory, with rapid and extensive metastasis causing many deaths.

Although the mechanism of invasion and metastasis remains controversial, Liotta et al. (1983) have proposed a three-step hypothesis for tumour cell invasion of the basement membrane (BM) and focused on the interaction of metastatic tumour cells with the extracellular matrix. In particular, proteinases in their second step have been a major interest for many oncologists, and numerous proteinases including type IV collagenase, urokinase type plasminogen activator and cathepsins, have been investigated (Mignatti et al. 1993).

Many studies have focused on the role of cathepsin B (CB) in tumour invasion and metastasis (Sloane and Kenneth 1984; Sloane 1990). In these studies, some investigators referred to CB in tumour cells as "CB-like proteinase" whose major difference from normal lysosomal CB is a shift in the pH activity profile. However, as anti-human CB antibody cannot discriminate between CB and CB-like proteinase, we designated both entities as CB in this study. In most previous studies on tumour CB, animal tumours or cancer cell lines were used but several human tumours including breast cancer (Recklies et al. 1980), pancreatic cancer (Rinderknecht and Renner 1980), and colonic cancer (Murnane et al. 1991) were investigated. However, little is known about human lung cancer and only Krepela et al. (1990) have reported that the activity of CB in the tumour tissue was elevated over that in lung parenchyma in all patients with primary lung adenocarcinoma. No obvious correlation was found between the tissue level of CB activity and the stage of primary lung tumour disease.

In this study, we examined the relationship between the immunohistochemical distribution of CB in surgically resected human lung adenocarcinoma specimens. Clinicopathological evaluations such as pathological stage, T factor, N factor, the grade of tumour differentiation and prognosis were made. We also investigated the histological relationship between CB and BM antigens using an immunohistochemical method.

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Materials and methods

The study specimens consisted of 64 human lung adenocarcinomas surgically resected at Hokkaido University Hospital between 1975 and 1985. All specimens were fixed in 10% formalin and embedded in paraffin. The patients were composed of 30 men and 34 women ranging in age from 31 to 83 years (mean 59.7 years). According to the stage classification of the International Union Against Cancer, they were classified into 25 patients with stage I, 8 with stage II, 29 with stage IIIA, and 2 with stage IIIB.

Polyclonal sheep anti-human CB antiserum was purchased from The Binding Site Ltd (Birmingham, England), and the specificity of the antiserum was confirmed by Western blotting and immunodiffusion experiment as previously described (Burnett and Stockley 1985; Nakamura et al. 1991). The monoclonal rabbit anti-human type IV collagen (CIV) antibody and the polyclonal rabbit anti-mouse laminin (LM) antibody were purchased from Advance Ltd (Tokyo, Japan) and E–Y Laboratories Inc. (San Mateo, Calif., USA), respectively.

Paraffin embedded sections 4 μm thick were stained by the avidin-biotin complex (ABC) immunoperoxidase technique. Briefly, the sections were deparaffinized and rehydrated through xylene and alcohol to phosphate buffered saline (PBS). The sections were incubated with 3% hydrogen peroxide to block endogeneous peroxidase activity, with normal serum for 20 min to block non-specific staining, and then with sheep anti-human CB (1:150) in a humid chamber at 4° C overnight. The sections were incubated with biotinylated rabbit anti-sheep antibody (Vector Laboratories, Burlingame, Calif., USA) for 30 min and with the Vectastain ABC Reagent (Vector) for 20 min. The colour was developed with 3,3'-aminobenzidine and hydrogen peroxide, and counter-stained with haematoxylin. Controls samples included the replacement of the primary specific antibody with normal sheep IgG or PBS.

CIV staining of serial sections was performed in a similar manner with the addition that deparaffinized sections were incubated with 0.4% pepsin at 37° C for 1 h. Rabbit monoclonal anti-human CIV antibody (1:100) was used as the primary antibody, and a biotinylated rabbit anti-mouse antibody was used as the secondary antibody. Controls samples included the replacement of the primary specific antibody with normal rabbit IgG or PBS. LM staining (antibody dilution; 1:100) was performed in a similar manner.

In the prognostic study, patients who died within 4 weeks after surgery or those who died of causes other than lung cancer within 5 years after surgery were excluded. Survival was calculated from the day of surgery. The survival curves of the patients were drawn using the Kaplan-Meier method, and the statistical evaluation was carried out using the generalized Wilcoxon test. The p values and significance of data were checked by Chi-square test.

Results

Although Howie et al. (1985) reported that normal ciliated epithelium in bronchi showed strong staining of CB, it revealed localized staining of CB at the apical edge of cells in this study (Fig. 1). Cilia, lymphocytes and the mucus in goblet cells appeared negative in both studies. In lung adenocarcinoma cells, some tumour cells showed granular cytoplasmic staining, and others none. The distribution of CB was estimated by counting only the number of cancer cells showing a granular cytoplasmic staining and was expressed as a percentage of the total number of cancer cells examined (1000 cells per specimen) by three independent observers (N.S., S.A., and S.O.). Tumour heterogeneity existed in 25% of 64

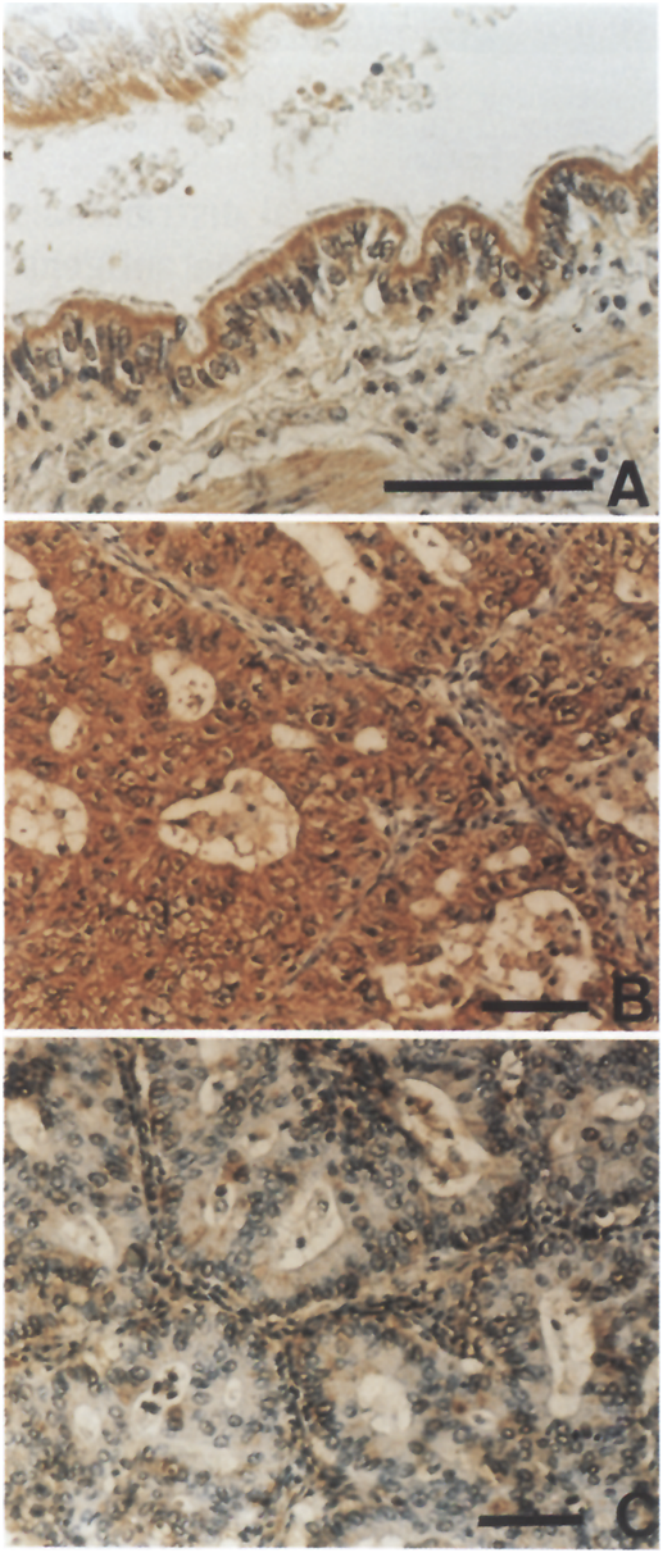

Fig. 1A–C Immunostaining for cathepsin B in normal bronchial epithelium and moderately differentiated adenocarcinoma of the lung. **A** Normal bronchial epithelium revealed strong staining of cathepsin B, but revealed localized staining of cathepsin B at the apical edge (X400). **B** + +, Diffuse cytoplasmic staining pattern for cathepsin B in 100% of tumour cells (X200). **C** −, Diffuse staining pattern for cathepsin B throughout cytoplasm in nearly 0% of tumour cells (X200). Scale bars = 150 μm

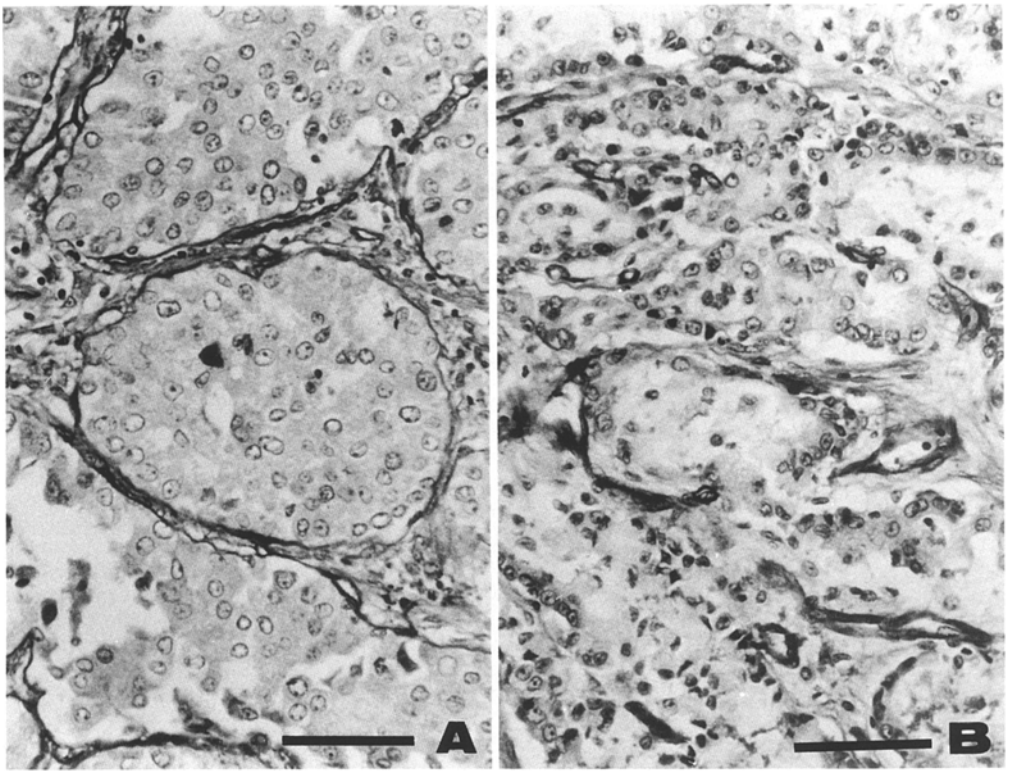

Fig. 2A, B Immunostaining for type IV collagen in moderately differentiated adenocarcinoma of the lung. **A** C (continuous pattern), Continuous immunoreactivity in more than 50% field of the tumour (×400). **B** D (discontinuous pattern), Discontinuous or fragmentary immunoreactivity of type IV collagen at tumour-associated BM in more than 50% field of the tumour (×400). Scale bars = 150 μm

cases. When tumour heterogeneity existed, we examined 1000 tumour cells each at 5 fields and expressed those average as the distribution of CB. The extent of cytoplasmic reactivity was divided into three groups, as follows: (−), staining of 0 to 19% of the cells; (+), staining of 20 to 79% of the cells; (++), staining of 80 to 100% of the cells. Control sections without the specific antibody showed negative staining (data not shown).

BM immunostaining was done with both CIV and LM. In almost all cases, using two serial sections, the staining pattern of CIV was identical to that of LM as previously reported by Barsky et al. (1983); therefore we simplify the discussion by referring only to CIV staining (Fig. 2.)

Normal bronchial epithelium and endothelium showed a well-defined continuous presence of CIV along its basement membranes. Some cancers showed a well-defined continuous presence of both CIV along their BMs, and others showed a discontinuous or fragmentary presence. Staining patterns of CIV were assessed according to staining continuity and divided into two groups, as follows: C (continuous pattern), continuous immunoreactivity of CIV at tumour-associated BM in more than 50% field of the tumour; D (discontinuous pattern), discontinuous or fragmentary immunoreactivity in more than 50% field of the tumour. Control sections without the specific antibody showed negative staining (data not shown).

Of 29 patients without lymph node metastasis: N(−) (i.e. N0), 17 (59%) had an immunoreactivity of (−) or (+), 12 (41%) had an immunoreactivity of (++); of 35 with lymph node metastasis: N(+) (i.e. N1, or N2 or N3), 9 (26%) had an immunoreactivity of (−) or (+), 26 (74%) had an immunoreactivity of (++). A significant difference was noted in the immunoreactivity between N (−) versus N (+) as determined by the Chi-square test ($p < 0.01$) (see Table 1).

Table 1 Relationship between staining pattern of cathepsin B and lymph node metastasis

Lymph node metastasis	Staining pattern of cathepsin B			(++)/total
	(−)	(+)	(++)	
N(−)	17 (7 + 10)		12	41%*
N(+)	9 (4 + 5)		26	74%*
	26 (11 + 15)		38	

* $p < 0.01$ by the Chi-square test

Of 64 patients, 44 were examined in regard to both CB and BM antigens. Of 18 with a continuous pattern of CIV, 5 (28%) had an immunoreactivity of (++). However, of 26 with a discontinuous pattern of CIV, 20 (77%) had an immunoreactivity of (++). There was a significant correlation between staining pattern of CIV and that of CB ($p < 0.01$) (Table 2).

Of 15 patients with well-differentiated adenocarcinomas, 15 (72%) had an immunoreactivity of (−) or

Table 2 Relationship between staining patterns of cathepsin B and type IV collagen

Staining pattern of type IV collagen	Staining pattern of cathepsin B			(++)/total
	(−)	(+)	(++)	
Continuous	13 (7 + 6)		5	28%*
Discontinuous	6 (0 + 6)		20	77%*
Total	19 (7 + 12)		25	

* $p<0.01$ by the Chi-square test

Table 3 Relationship between staining pattern of cathepsin B and the grade of tumour differentiation

The grade of differentiation	Staining pattern of cathepsin B			(++)/total
	(−)	(+)	(++)	
Well	15 (5 + 10)		6	28%*
Moderate	8 (4 + 4)		23	74%*
Poor	2 (1 + 1)		10	83%*
Total	25 (10 + 15)		39	

* $p<0.01$ by the Chi-square test

(+), 6 (28%) had an immunoreactivity of (++); of 25 with moderately-differentiated adenocarcinomas, 8 (26%) had an immunoreactivity of (−) or (+), 23 (74%) had an immunoreactivity of (++); of 12 with poorly differentiated adenocarcinomas, 10 (83%) had an immunoreactivity of (++). A significant difference was noted in the immunoreactivity among the grade of tumour differentiation as determined by the Chi-square test ($p<0.01$) (Table 3).

Fifty-eight patients were enrolled in the prognostic study of CB staining pattern. The patients were divided into three groups: 9 patients with CB (−) tumours, 13 patients with CB (+) tumours and 36 patients with CB (++) tumours. Both the patients with CB (−) tumours and those with CB (+) tumours had significantly longer survival times than those with CB (++) tumours ($p<0.01$). However, the patients with CB (−) tumours and those with CB (+) tumours did not have significantly different survival times (Fig. 3.).

We also performed a prognostic analysis with particular respect to the distribution of CB and the grade of tumour differentiation. In well-differentiated adenocarcinomas, CB (++) was associated with a significant prognostic disadvantage [5-year survival time; CB (++) 25%, CB (−) or (+) 92%, $p<0.05$]. The same

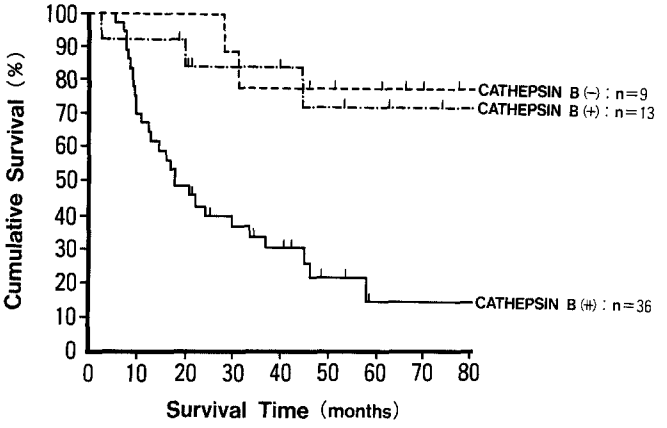

Fig. 3 Survival curves of the patients with lung adenocarcinoma stratified by the staining pattern of cathepsin B. Both the patients with cathepsin B (−) tumours and those with cathepsin B (+) tumours had significantly longer survival times than those with cathepsin B (++) tumours ($p<0.01$). But the patients with cathepsin B (−) tumours and those with cathepsin B (+) tumours did not have significantly different survival times

association was seen in moderately differentiated adenocarcinomas [5-year survival time; CB (++) 18%, CB (−) or (+) 52%, $p<0.05$].

Discussion

To our knowledge, this study is the first to examine the existence of both CB and BM antigens in human lung adenocarcinomas by immunohistochemical methods.

CB is synthesized in the rough endoplasmic reticulum, processed in the Golgi apparatus and delivered to the lysosomes in normal cells. It plays an important role in the catabolism of various intracellular proteins in lysosomes, and has been implicated in extracellular matrix degradation in disease states such as tumour metastasis (Erdel et al. 1990), muscular dystrophy (Kominami et al. 1987), emphysema (Burnett and Stockley 1985), and arthritis (Etherington et al. 1988) recently. CB in tumour cells, directly by degrading LM (Lah et al. 1989), proteoglycan (Morrison et al. 1973) and collagen (Burleigh et al. 1974), or indirectly through the activation of procollagenase (Eeckhout and Vaes 1977) and proenzyme uPA (Kobayashi et al. 1992), has been found to be involved in the degradation of extracellular matrix such as BMs besides cellular protein turnover. In tumour CB, Sloane et al. (1981) reported that the activity of the lysosomal CB is significantly elevated in a variant of the B16 melanoma with high metastatic potential. In recent studies, by enzymatic assays of CB in subcellular fractions in tumour cells, its activity in plasma membrane fraction increased in correspondence with metastatic potential (Sloane et al. 1986). And its activity of invasive cells was higher than that of non-invasive cells in the lysosomal fraction as well as in the plasma membrane fraction (Weiss et al. 1990). These studies

suggest that both CB in lysosome and in plasma membrane are important in tumour invasion and metastasis. Although the study of localization by immunohistochemical staining and activity of CB is rare (Keppler et al. 1988; Weiss et al. 1990), it is considered that tumour cells with diffuse cytoplasmic staining pattern have enriched CB, indicating increased CB in lysosome and/or in plasma membrane. Interestingly, most of the positively stained tumour cells in our study appeared fine granular staining throughout cytoplasm, which appeared to be called as "diffuse cytoplasmic staining pattern" in Keppler's study. As Keppler and his associates reported, the component giving a diffuse cytoplasmic staining of tumour cells may be tumour-associated CB-like proteinase.

In our study, the distribution of CB lacks a significant association with pathological stage and T factor (data not shown). It did, however, have a significant relationship with lymph node metastasis, the grade of tumour differentiation and prognosis. Lung adenocarcinomas with CB (++) had a higer risk of lymph node metastasis, poorer differentiation and a worse prognosis than tumours which were CB (−) or (+). However, the distribution of CB was not simply related to the grade of differentiation. The distribution of CB has a relationship to lymph node metastasis, and may be used as an prognostic marker in human lung adenocarcinoma.

Basement membranes are thought to be barriers against tumour invasion and metastasis, and their essential components are CIV and LM. As CB has been shown to degrade collagen and LM in vitro, we investigated the distribution of CB and BM antigens immunohistochemically using serial sections. In our study, lung adenocarcinomas with CB (++) had a higer risk of BM degradation than tumours which were CB (−) or (+). This result also suggests that CB plays a role in tumour invasion and metastasis. In the past, many investigations have shown close relationship between the distribution of BM antigens, the incidence of distant metastasis and patient survival times (Foster et al. 1984; Daher et al. 1987). Nakajima (1991) has also showed that discontinuous pattern of CIV was associated with lymph node metastasis and with shorter survival times in lung adenocarcinoma. From these investigations and from our study, we may speculate that CB influences the mortality of patients with lung adenocarcinomas mainly by degrading extracellular matrix such as BMs.

There were no differences between CB (−) tumours and CB (+) tumours with regard to prognosis and other factors. We cannot explain the reasons for this, but we speculate that this may be due to the down-regulation of tumour cell-secreted CB activity by cysteine proteinase inhibitors such as the stefin and cystatin families (Sloane 1990). Further studies are needed to establish the complex interplay between CB and its inhibitors.

Our study suggests that the immunohistochemical distribution of CB will be a useful indicator for the evaluation of invasion, metastasis and prognosis in human lung adenocarcinoma.

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